Review

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Spatiotemporal regulation of posttranslational modifications in the DNA damage response

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Abstract

A timely and accurate cellular response to DNA damage requires tight regulation of the action of DNA damage response (DDR) proteins at lesions. A multitude of posttranslational modifications (PTMs) of chromatin and chromatin-associated proteins coordinates the recruitment of critical proteins that dictate the appropriate DNA repair pathway and enable the actual repair of lesions. Phosphorylation, ubiquitylation, SUMOylation, neddylation, poly (ADP-ribosyl)ation, acetylation, and methylation are among the DNA damage-induced PTMs that have taken center stage as important DDR regulators. Redundant and multivalent interactions of DDR proteins with PTMs may not only be a means to facilitate efficient relocalization, but also a feature that allows high temporal and spatial resolution of protein recruitment to, and extraction from, DNA damage sites. In this review, we will focus on the complex interplay between such PTMs, and discuss the importance of their interconnectivity in coding DNA lesions and maintaining the integrity of the genome.

Keywords chromatin; DNA repair; Poly(ADP-ribosyl)ation; SUMO; ubiquitin
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See the Glossary for abbreviations used in this article.

Introduction

When the integrity of the genome is being challenged by intrinsic and extrinsic insults that cause DNA damage, it is of paramount importance for cells to take appropriate action. The first and most critical step is the recognition of DNA lesions, which can be highly diverse in nature, ranging from DNA single-strand breaks (SSBs) to DNA double-strand breaks (DSBs) to light-induced base damage (photolesions) to protein–DNA adducts. The communality between these lesions is the serious consequence for cellular homeostasis that their presence can have. Since failure to maintain genome integrity is directly linked to various human disorders such as cancer, neurodegeneration, and immunodeficiency (Jackson & Bartek, 2009; Lord & Ashworth, 2012), the cellular response to DNA damage has received a great deal of attention. However, despite the wealth of knowledge that has been gathered on this topic, there is still much that needs to be learned before we can grasp the enormous complexity of the DNA damage response (DDR). One important point that has become increasingly clear in the course of studying the signaling and repair pathways involved in harnessing the cellular environment against DNA damage is their highly integrative nature (Lukas *et al*, 2011; Marteijn *et al*, 2014).

DNA damage recognition is instantly followed by the activation of a complex signaling cascade, which marks the lesions, coordinates cell cycle progression, and activates the desired DNA repair pathways. Posttranslational modifications (PTMs) that are triggered by the presence of damaged DNA play a central role in the initiation and execution of these cellular responses. Bearing witness to the importance of PTMs during genotoxic stress is a plethora of studies on selective protein phosphorylation and dephosphorylation triggered by DNA damage. However, while protein phosphorylation has for long been in the limelight as the central DNA damage-induced modification (Shiloh, 2003), the picture has become far more complex with the realization that phosphorylation is just one of a variety of DDR-regulating PTMs. Recent years have seen the implication of a number of other covalent modifications, including those involving conjugation of proteinaceous modifiers such as ubiquitin and the ubiquitin-like molecules SUMO and Nedd8. DNA damage furthermore induces various other modifications, such as poly(ADP-ribose) (PAR) and acetyl and methyl groups, which facilitate recruitment of proteins. The broad spectrum of PTMs at DNA lesions brings up questions not only about the molecular mechanisms that regulate their co-existence, but also with regard to the functional significance of having such a complex integrated network in place to defend the integrity of the genome.

In this review, we will discuss our present understanding of this complex network of pathways, with special attention to the PTM crosstalk and interconnectivity that directs the wiring of these pathways in response to DNA damage. Altogether, the body of data on the central role of these modifications suggests that we are not dealing with a cacophony of simultaneously occurring modifications, but rather with an orchestrated process that safeguards a proper and timely response of cells to minimize the potential devastating effects of compromised genome integrity, while at the

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Glossary

BRCT	BRCA1 C-terminal
CRL	cullin-RING ubiquitin ligase
CSN	COP9 signalosome
DDR	DNA damage response
DSB	DNA double-strand breaks
FHA	Fork head-associated
HR	homologous recombination
ICL	DNA interstrand cross-link
MIU	motif interacting with ubiquitin
NER	nucleotide excision repair
NHEJ	non-homologous end joining
PAR	poly(ADP-ribose)
PRC	polycomb repressive complex
PTM	posttranslational modification
SIM	SUMO-interacting motif
SSB	DNA single-strand breaks
STUbL	SUMO-targeted ubiquitin ligase
TLS	translesion synthesis
UBZ	ubiquitin-binding zinc finger
UDR	ubiquitin-dependent recruitment motif
UIM	ubiquitin-interacting motif
UV	ultraviolet
γH2AX	histone variant H2AX phosphorylated at serine 139

same time preventing inaccurate activation of such responses. We will illustrate the principles of PTM interplay mainly, but not exclusively, with examples from the cellular response to DSBs. We would like to stress, however, that similar phenomena are frequently found in other DDR pathways. In particular, nucleotide excision repair (NER) of photolesions (van Cuijk *et al*, 2014) and damage tolerance by translesion synthesis (TLS) (Jansen *et al*, 2015) are examples of other pathways tightly regulated by multiple PTMs. While referring the reader to excellent recent reviews for a detailed overview of the distinct cellular responses to DNA damage (Lukas *et al*, 2011; Polo & Jackson, 2011; Chapman *et al*, 2012; Parsons & Dianov, 2013; Caldecott, 2014; Dijk *et al*, 2014; Jansen *et al*, 2015), we will here focus on the general underlying principles of DNA damage-induced PTMs and speculate on the advantage of using multiple parallel and interconnected signaling cascades in the DSB response.

PTM-guided PTMs

A common principle in the regulation of DNA damage-induced PTMs is vectorial pathways, meaning that one modification directly stimulates or represses the generation of another modification. These regulatory circuits connect the large spectrum of PTMs in an integrated network, in which their appearance and disappearance are directly linked in space and time. This does not only apply to signaling pathways activated by various types of DNA lesions, but is a widespread phenomenon in cellular regulatory cascades. One of the best-described examples is the interplay between phosphorylation and ubiquitylation in regulating proteasomal degradation, where phospho-modifications can either generate a "phosphodegron", a recognition signal leading to ubiquitylation and proteasomal degradation, or on the contrary prevent interaction between a ubiquitin ligase and target protein, which will therefore be stabilized (Ravid & Hochstrasser, 2008). While crosstalk between phosphorylation and ubiquitylation is also central to the DDR (Brinkmann *et al*, 2015), this paradigm of PTM crosstalk plays a much broader role in the DDR and also applies to other kinds of modifications, thus generating a complex network. Although histone modifications are integrated in this process as well (Smeenk & van Attikum, 2013), DDR coordination includes also modifications of many other proteins that transiently associate with chromatin. Conceptually, these signaling cascades resemble each other in the sense that they are based on PTM-guided modifiers, that is, enzymes that are recruited to sites of DNA damage through the interaction with specific PTMs, upon which they subsequently either introduce or revert another modification. Such a mechanism may ensure that proteins are both recruited in an ordered fashion and removed again in a timely manner after damage repair.

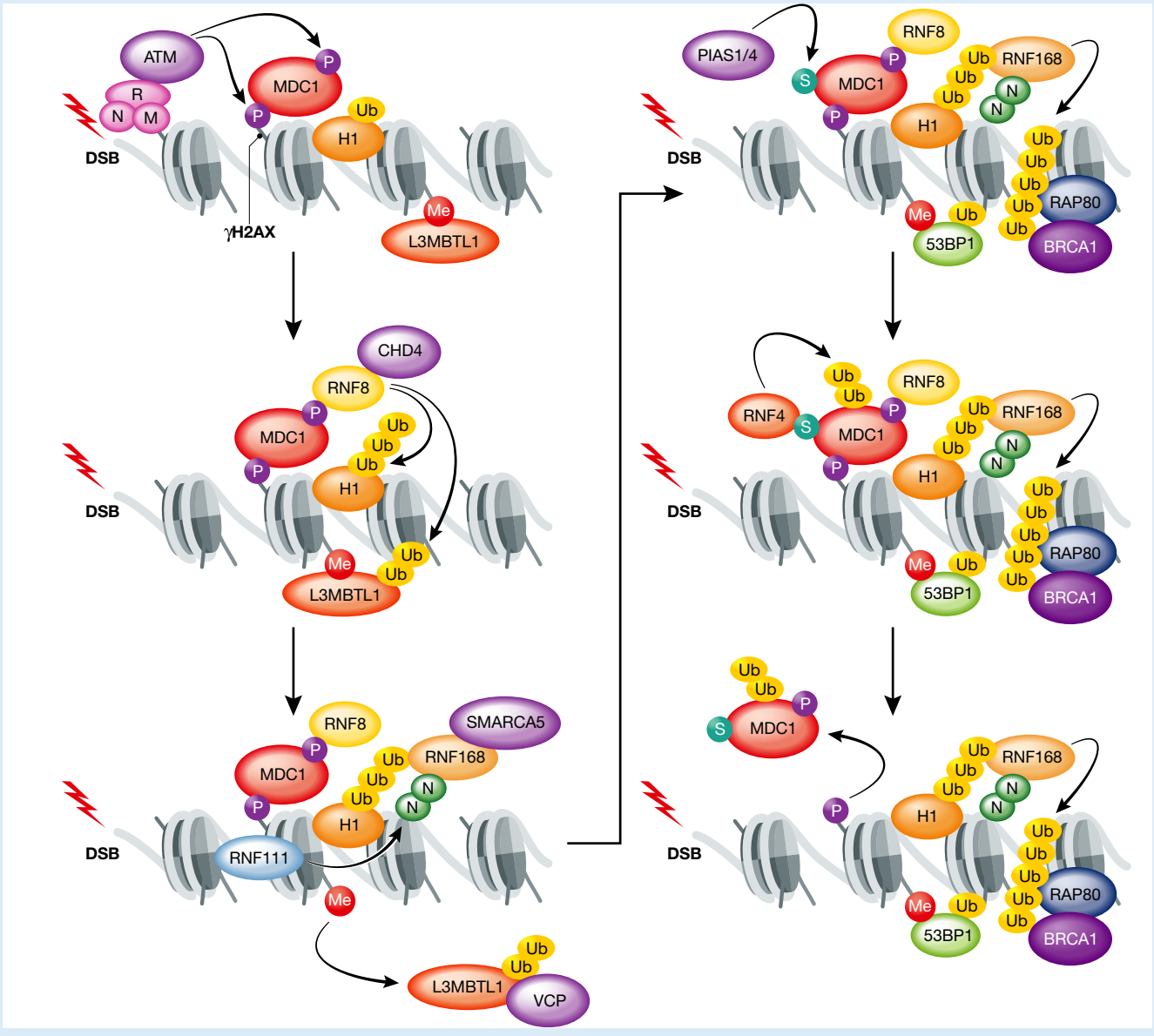
Phospho-guided ubiquitylation

Similar to the above-mentioned example of phosphodegrons in protein degradation, phosphorylation is also a master regulator of DNA damage-induced ubiquitylation, although the outcome during the DDR is not necessarily proteolysis. The phosphatidylinositol-3-kinase-related kinases ATM, ATR, and DNA-PK are responsible for the induction of local phosphorylation at sites of DNA damage. While the targets of these kinases are numerous, one of the critical chromatin proteins phosphorylated is the histone variant H2AX. In addition to H2AX phosphorylation (termed γH2AX), several studies also revealed changes in the ubiquitylation status of core histones in response to ultraviolet (UV) light-induced DNA damage (Bergink *et al*, 2006; Kapetanaki *et al*, 2006; Wang *et al*, 2006). At least in the context of DSBs, DNA damage-induced ubiquitylation is directly linked to γH2AX, as revealed with the identification of the ubiquitin ligase RNF8 as a central mediator of chromatin-associated ubiquitylation (Huen *et al*, 2007; Kolas *et al*, 2007; Mailand *et al*, 2007). This pathway, triggered by the initial γH2AX-dependent recruitment of RNF8, forms a paradigm for the multilayered interplay between PTMs in the cellular response to DNA damage, to which we will come back repeatedly in this review to illustrate the various concepts (see Box 1).

RNF8 contains both a RING domain (required for conjugation of ubiquitin to target proteins) and a phospho-threonine residue-binding fork head-associated (FHA) domain, known to facilitate DNA damage-induced interactions with ATM/ATR/DNA-PK substrates (Stracker *et al*, 2004; Stucki & Jackson, 2006). Accordingly, the FHA domain of RNF8 is responsible for its ATM-dependent relocalization to DSBs by mediating interaction of RNF8 with phosphorylated MDC1, which itself selectively binds γH2AX (Huen *et al*, 2007; Kolas *et al*, 2007; Mailand *et al*, 2007). Upon binding to phosphorylated MDC1, RNF8 facilitates local ubiquitylation, triggering a series of downstream events that result in the recruitment of proteins involved in DNA damage signaling and DNA repair (Fig 1A). Sequestration of RNF8 appears to be sufficient, as even artificially tethering RNF8 to chromatin can induce a chromatin-associated ubiquitylation response resembling the one observed at DSBs (Luijsterburg *et al*, 2012a). Interestingly, RNF8 is also recruited to UV-induced photolesions in an ATR- and MDC1-dependent manner, but in this case independent of γH2AX (Marteijn *et al*, 2009). Thus, the phospho-recruited ubiquitin ligase RNF8 links the ATM/ATR phosphorylation response to local protein

Box 1: DNA double-strand break-induced ubiquitylation response

The DSB-induced ubiquitylation response incorporates various PTMs and provides a paradigm for the integrated nature of the networks involved in the signaling and repair of these lesions. The MRE11-RAD50-NBS1 (MRN) complex detects DSBs and associates with ATM kinase to facilitate ATM recruitment and ATM-dependent phosphorylation (P) of histone H2AX (forming "γH2AX") in DSB-flanking chromatin. Via its γH2AX-binding C-terminal BRCT domain, MDC1 is recruited to γH2AX and subsequently phosphorylated by ATM. The ubiquitin ligase RNF8 interacts with phosphorylated MDC1 and ubiquitylates H1-type linker histones responsible for RNF168 recruitment, as well as a number of other proteins, including L3MBTL1 bound at methylated histone H4. Following recruitment to DSBs, the RNF111 E3 ubiquitin ligase neddylates histone H4 in DSB-flanking chromatin, which together with ubiquitylated histone H1 recruits the RNF168 ubiquitin ligase via its MIUs. Once bound, RNF168 decorates DSB-flanking chromatin with ubiquitin conjugates, an event that is stimulated by RNF8- and RNF168-associated chromatin remodelers CHD4 and SMARCA5/SNF2H. In addition, RNF168 is responsible for recruitment of VCP/p97, which removes RNF8-ubiquitylated L3MBTL1 from methylated histone H4. 53BP1 then accumulates in the vicinity of DSBs by simultaneously binding RNF168-induced monoubiquitin conjugates on histone H2A(X) and methylated histone H4. In addition, RNF168-induced ubiquitin conjugates also facilitate relocalization of BRCA1 through its binding partner RAP80, which binds ubiquitin chains via its tandem ubiquitin-interacting motifs (UIM). MDC1 residence time is regulated through SUMOylation (S) by PIAS1/4. SUMOylated MDC1 recruits the SUMO-targeted ubiquitin ligase (STUbL) RNF4, which can target MDC1 for ubiquitylation and extraction from chromatin. Me, methyl group; N, Nedd8 moiety; P, phosphate group; S, SUMO moiety; Ub, ubiquitin moiety.



ubiquitylation at DSBs and UV lesions. Ultimately, RNF8-dependent chromatin ubiquitylation around sites of DNA damage culminates in the recruitment of DNA repair proteins such as 53BP1 and BRCA1 (Huen *et al*, 2007; Kolas *et al*, 2007; Mailand *et al*, 2007; Marteijn *et al*, 2009), which will be discussed in more detail in the section "Ubiquitin-guided ubiquitylation".

More recent work identified DSB recruitment of another ubiquitin ligase, the RNF20/RNF40 heterodimer, which induces histone H2B monoubiquitylation on lysine 120 (Nakamura *et al*, 2011). This H2BK120ub modification, usually associated with active gene transcription (Weake & Workman, 2008), in turn causes increased lysine 4 methylation of histone H3 (H3K4me) and recruitment of the SMARCA5/SNF2H chromatin remodeler, resulting in chromatin decondensation to allow efficient assembly of DSB repair factors and stimulate removal of DNA breaks from the genome (Nakamura *et al*, 2011). Importantly, also this cascade of events is triggered by ATM kinase activity, which is required for the phosphorylation and subsequent recruitment of RNF20/RNF40 to DSBs (Moyal *et al*, 2011). These findings exemplify yet another phospho-dependent ubiquitylation event that is critical for a proper DSB response.

Ubiquitin-guided ubiquitylation

Given that ubiquitin modifications vary between monoubiquitylation and polyubiquitylation, and in the latter case also with regard to the type of linkage connecting individual ubiquitin molecules (Pickart, 2000), it is oversimplified to look upon ubiquitylation as one single modification. Considerable effort has been invested in uncovering a "ubiquitin code" that translates given types of ubiquitin modifications into specific functional consequences for the modified protein (Komander & Rape, 2012). Although our understanding of this complex code is still rudimentary, some basic principles are starting to emerge. For example, ubiquitin chains linked by conjugating the carboxy-terminus of one ubiquitin molecule to lysine residue 48 in the preceding ubiquitin (K48-linked ubiquitin chains) are long known as the canonical targeting signal for proteasomal degradation (Hershko & Ciechanover, 1998). Recent data indicate that most other ubiquitin chain types can also serve as proteasome-targeting signals (Kravtsova-Ivantsiv & Ciechanover, 2012), however with the notable exception of K63-linked polyubiquitin, which instead fulfills exclusively non-proteolytic functions (Zhao & Ulrich, 2010). The realization that BRCA1 localization to DSBs depends on K63-linked chains implicated this particular type of ubiquitin modification in the DDR (Sobhian *et al*, 2007; Huang *et al*, 2009). Moreover, the requirement of Ubc13, a ubiquitin-conjugating enzyme generating K63 linkages (Pickart, 2001), for recruitment of RAD18 and its binding partner RAD51C, a key factor involved in homologous recombination (HR), suggested an important role for K63-linked ubiquitin chains in DSB repair (Zhao *et al*, 2007). More recently, also K27-linked ubiquitin chains were found on chromatin at DSBs. These chains were generated by the ubiquitin ligase RNF168 by targeting histones H2A and H2AX (Gatti *et al*, 2015). Similar to K63-linked ubiquitin, K27-linked chains are important for the recruitment of DSB repair factors such as 53BP1, RAP80, and BRCA1 (Gatti *et al*, 2015). How these different types of ubiquitin chains cooperate to orchestrate a robust DSB response needs to be addressed in future work.

RNF8 was initially found to be required for K63-linked ubiquitylation of histone H2A (Huen *et al*, 2007; Kolas *et al*, 2007; Mailand *et al*, 2007), but the identification of RNF168 as a second ubiquitin ligase that cooperates with RNF8 to generate K63-linked ubiquitin chains (Doil *et al*, 2009; Stewart *et al*, 2009) eventually revealed the underlying multistep process that relies on ubiquitin-guided ubiquitylation (Mattiroli *et al*, 2012). RNF168 contains ubiquitin-binding domains known as MIUs (motif interacting with ubiquitin) that mediate its binding to the ubiquitin conjugates synthesized by RNF8 (Doil *et al*, 2009; Stewart *et al*, 2009) (Fig 1B). Although interaction with monoubiquitylated histone H2A was originally assumed to facilitate RNF168 recruitment, more recent data showing that RNF8 cannot ubiquitylate H2A within nucleosomes suggested that another chromatin-associated RNF8 target facilitates sequestration of RNF168 (Mattiroli *et al*, 2012). Indeed, H1-type linker histones have recently been identified as the critical substrate of RNF8 that facilitates recruitment of RNF168 (Thorslund *et al*, 2015). While H1 linker histones contain many lysine residues, several of which have been found to be modified with ubiquitin under basal conditions, a dramatic increase in K63 polyubiquitylation of these histones is observed at DSBs in a manner dependent on the concerted action of RNF8 and Ubc13 (Thorslund *et al*, 2015). It is tempting to speculate that RNF8 requires the basal ubiquitin modifications on H1 to establish the K63 polyubiquitin mark since Ubc13, which is known to efficiently extend K63-linked ubiquitin chains, seems less well adapted for conjugating the initial ubiquitin to substrates (Petroski *et al*, 2007; Windheim *et al*, 2008). Remarkably, although RNF168 when bound to polyubiquitylated histone H1 monoubiquitylates lysines 13 and 15 of histone H2A and H2AX, it still requires RNF8 to generate K63-linked ubiquitin chains (Gatti *et al*, 2012; Mattiroli *et al*, 2012). Also here, the tendency of Ubc13 to elongate preexisting ubiquitin marks with K63-linked chains may explain the need for a concerted action of RNF8 and RNF168 in this process, since RNF8 may generate such chains using monoubiquitylated H2A and H2AX generated by RNF168 as substrates. Interestingly, RNF168-mediated ubiquitylation in particular appears to be rate limiting in the cascade, explaining why this event is tightly controlled at various levels. For example, two ubiquitin ligases, Ubr5 and Trip12, constitutively moderate the steady-state protein levels of RNF168, with their depletion resulting in supra-physiological accumulation of RNF168 and downstream DDR factors, compromising proper DNA repair (Gudjonsson *et al*, 2012). Moreover, the RNF168 paralog RNF169 also counteracts RNF168-mediated recruitment of DDR proteins, as discussed below (Poulsen *et al*, 2012).

Notably, RNF8 also interacts with ubiquitin-conjugating enzymes that generate canonical proteasome-targeting K48-linked ubiquitin chains (Ito *et al*, 2001). Consistently, K48-linked ubiquitin chains are synthesized at DSBs in an RNF8-dependent fashion (Feng & Chen, 2012; Lok *et al*, 2012), but unlike the K63-linked chain modification of chromatin proteins (Huen *et al*, 2007; Mailand *et al*, 2007), this K48-linked polyubiquitylation response occurs rapidly and more transiently after DSB recognition (Ramadan, 2012). This initial wave of K48-linked ubiquitin chains generated by RNF8 may also be involved in RNF168 recruitment, whose MIUs can bind both K63- and K48-linked ubiquitin chains (Feng & Chen, 2012). Furthermore, the ability of RNF8 to bind Ubc13 and synthesize K63-linked chains is selectively stimulated by the ubiquitin ligase HERC2 (Bekker-Jensen *et al*, 2010), whose interaction with RNF8 is promoted by SUMOylation (Rendtlew Danielsen *et al*, 2012). Therefore, SUMOylation may regulate the relative balance between K48- and K63-linked ubiquitylation mediated by RNF8. However, further studies are required to firmly establish the DDR role of HERC2, which unlike RNF8 and RNF168 is dispensable for DNA damage-induced ubiquitylation in avian cells (Oestergaard *et al*, 2012). Disappearance of K48-linked ubiquitin conjugates coincides with recruitment of

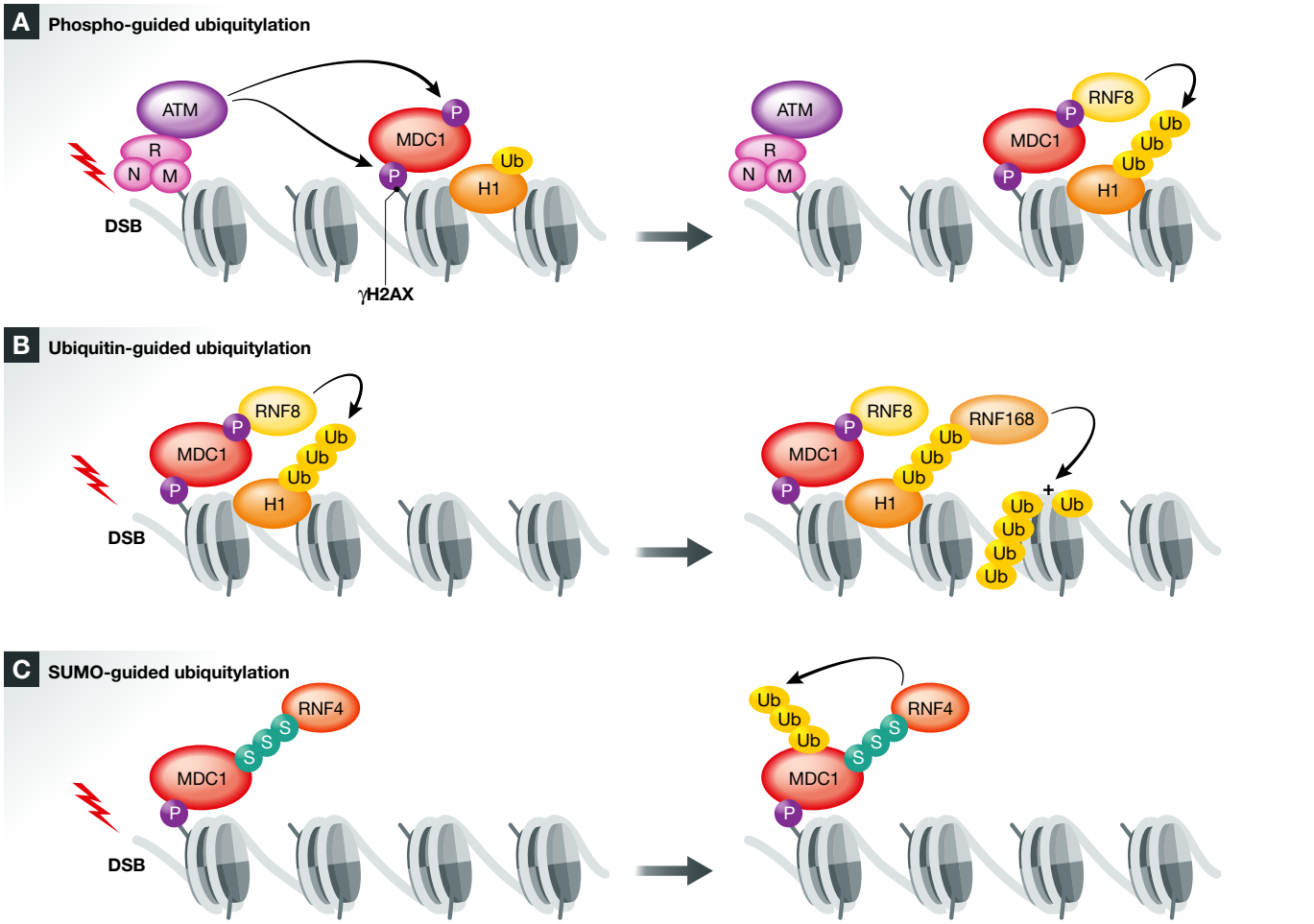


Figure 1. PTM-guided PTMs in the DDR (part 1).
Schematic representation of (A) phospho-guided ubiquitylation, (B) ubiquitin-guided ubiquitylation, and (C) SUMO-guided ubiquitylation. See sections "Phospho-guided ubiquitylation", "Ubiquitin-guided ubiquitylation" and "Ubiquitin-guided SUMOylation and SUMO-guided ubiquitylation" for more details. P, phosphate group; S, SUMO moiety; Ub, ubiquitin moiety.

VCP/p97 (Meerang *et al*, 2011), a ubiquitin-selective chaperone/segregase that has been shown to facilitate ubiquitin-dependent extraction of chromatin-associated proteins for proteasomal destruction or other purposes (Dantuma & Hoppe, 2012). Indeed, several RNF8 targets, such as the initiator of non-homologous end joining repair (NHEJ) Ku80 (Feng & Chen, 2012), the demethylase JMJD2A (Mallette *et al*, 2012), and the polycomb protein L3MBTL1 (Acs *et al*, 2011), were found to be extracted from chromatin and/or degraded by the proteasome following DNA damage, with direct VCP/p97 involvement confirmed at least for the cases of Ku80 and L3MBTL1 (Acs *et al*, 2011; Brown *et al*, 2015). Thus, the RNF8/RNF168 pathway illustrates the complex role of various types of proteolytic and non-proteolytic ubiquitin modifications in the DDR and shows that their occurrence and regulation are interconnected.

Ubiquitin-guided SUMOylation and SUMO-guided ubiquitylation

DNA damage-induced modifications with the small ubiquitin-like modifier SUMO have been reported for a number of proteins that are directly or indirectly involved in DNA repair (Sarangi & Zhao, 2015). One of the best studied examples is the replication clamp factor PCNA, which is subject to SUMOylation, monoubiquitylation, and K63-linked polyubiquitylation. At stalled replication forks in yeast, these mutually exclusive modifications play a key role in the decision either to bypass a lesion via error-prone translesion synthesis DNA polymerases, or to engage an error-free mechanism that involves template switching (Ulrich & Jentsch, 2000; Hoege *et al*, 2002; Stelter & Ulrich, 2003). However, the reach of SUMO modifications is more widespread. Recruitment of the PIAS family SUMO ligase Siz2 to single-stranded DNA at lesions catalyzes a wave of chromatin-associated protein SUMOylation in budding yeast (Psakhye & Jentsch, 2012). HR repair is compromised by genetic ablation of SUMOylation in general, but not of individual SUMO modification sites on target proteins, suggesting that mainly the sum of these SUMO modifications is critical for DNA repair (Psakhye & Jentsch, 2012). Collective group modification of DNA damage-associated proteins with SUMO may act as a molecular "glue" that stimulates protein–protein interactions, consistent with the observed broad spectrum of DSB-induced substrates, which in

addition are often SUMOylated at multiple sites (Psakhye & Jentsch, 2012). Such a generic stabilizing effect on protein interactions at sites of DNA damage may be a critical determinant for the regulatory role of this modification in DNA repair.

The role of SUMO in the DDR is however much more complex. In higher eukaryotes, SUMO is involved in both the coordinated recruitment and removal of proteins from DSBs, each time involving interplay between SUMOylation and DSB-induced protein ubiquitylation. All three human SUMO paralogs, SUMO1 and the poly-SUMO chain-forming SUMO2 and SUMO3, are heavily enriched at DSBs in a fashion that requires the SUMO ligases PIAS1 and PIAS4 (Galanty *et al*, 2009). Interestingly, the accumulation of SUMO conjugates at DSBs depends on RNF8/RNF168-mediated ubiquitylation (Galanty *et al*, 2009). Among the targets of DSB-induced SUMOylation are the DNA repair proteins MDC1, 53BP1, BRCA1, and RPA (Galanty *et al*, 2009, 2012; Luo *et al*, 2012; Yin *et al*, 2012), as well as the ubiquitin ligases RNF168 and HERC2 (Rendtlew Danielsen *et al*, 2012). The molecular basis of the RNF8 and RNF168 requirement for efficient SUMOylation at DSBs is presently not clear. Modification of the ubiquitin ligase HERC2 with SUMO1 is required to stabilize the interaction between RNF8 and Ubc13 allowing efficient K63-linked ubiquitylation (Rendtlew Danielsen *et al*, 2012), and consistently, it has been shown that SUMOylation is not only dependent on RNF8, but also required for a proper K63-ubiquitylation response at DNA breaks. Finally, proteomic analysis of proteins modified by SUMO2 upon DNA damage confirms that, in addition to the proteins mentioned above, a wide spectrum of chromatin-associated proteins is SUMOylated (Hendriks *et al*, 2015). Functional studies toward the role of these SUMOylated proteins may shed more light on the relevance of this modification during the DDR.

The picture becomes even more complex when considering the role of the SUMO-targeted ubiquitin ligase (STUbL) RNF4, which is known to regulate various cellular processes, in particular those related to genotoxic stress (Prudden *et al*, 2007; Sun *et al*, 2007) and proteotoxic stress (Lallemand-Breitenbach *et al*, 2008; Tatham *et al*, 2008). By selectively ubiquitylating SUMOylated substrates, RNF4 converts the SUMO modification into a targeting mark for proteasomal degradation. Central for this action are the presence of SUMO-interacting motifs (SIMs) in RNF4, which allow RNF4 to interact with a variety of SUMOylated proteins. In a SIM- and SUMO-dependent fashion, RNF4 also localizes to DSBs, where it regulates the timely removal and degradation of repair proteins such as MDC1 and RPA (Galanty *et al*, 2009; Luo *et al*, 2012; Yin *et al*, 2012). Persistent accumulation of MDC1 and RPA in the absence of RNF4 correlates with defective DSB repair by HR and NHEJ, which in case of MDC1 is directly linked to RNF4-dependent ubiquitylation of a certain lysine residue specifically required for HR to take place (Luo *et al*, 2012) (Fig 1C).

Although the most common outcome of RNF4-dependent ubiquitylation appears to be protein removal from chromatin, there are also data that support a role in ubiquitin-dependent protein recruitment. RAP80, a mediator protein critical for BRCA1 translocation to DSBs (Sobhian *et al*, 2007), contains a SIM that cooperates with its tandem ubiquitin-binding domains to mediate BRCA1 recruitment (Guzzo *et al*, 2012). *In vitro*, RAP80 binds preferentially to hybrid SUMO-ubiquitin chains, which have been proposed to be produced by RNF4 in a DNA damage-inducible manner (Guzzo *et al*, 2012). Finally, proteomic analyses confirm that RNF4 is involved at a global scale in both protein recruitment and extraction at DNA lesions (Hendriks *et al*, 2015). Notably, following DNA damage induction, RNF4 targets two related histone demethylases, JARID1B/KDM5B and JARID1C/KDM5C, for chromatin extraction and recruitment, respectively (Hendriks *et al*, 2015).

SUMO-targeted ubiquitylation is also involved in NER (Marteijn *et al*, 2014). A primary target in NER is the UV lesion sensor XPC, which in response to UV irradiation is modified with both SUMO2/3 conjugates and non-degradative polyubiquitin chains, the latter of which dependent on the STUbL RNF111 (Poulsen *et al*, 2013). RNF111-mediated XPC ubiquitylation controls timely removal of the recognition protein from UV lesions, granting downstream repair proteins access to the damage site (van Cuijk *et al*, 2015). Together, these examples show that SUMO and ubiquitin modifications can cross-interact in various ways, with SUMOylation depending on ubiquitylation as observed for RNF8 in the DSB response, and with ubiquitylation depending on SUMOylation as in the case of the STUbLs RNF4 and RNF111 during the DSB and the UV response, respectively.

Nedd8-guided ubiquitylation

Of all twelve ubiquitin-like modifiers, Nedd8 has the highest similarity to ubiquitin but like other ubiquitin-like proteins still relies on its own dedicated activation and conjugation enzymes (Jentsch & Pyrowolakis, 2000). Nedd8 is best known for its ability to modify cullin proteins, which belong to a family of molecular scaffolds that are core components of the CRL ubiquitin ligases (Petroski & Deshaies, 2005). Nedd8 conjugation (neddylation) to cullins stimulates ubiquitin ligase activity of their enzymatic complexes, making Nedd8 probably the ubiquitin-like protein that is most directly linked to the ubiquitylation machinery (Lydeard *et al*, 2013).

A recent screen identified Nedd8 as the only other ubiquitin-like modifier (in addition to ubiquitin and SUMO) that accumulated at DSBs. At these lesions histone H4 becomes polyneddylated in an RNF111-dependent manner (Ma *et al*, 2013). This suggests that histones may not only be primarily ubiquitylation substrates at DSBs. Remarkably, Nedd8 modification appears in turn required for efficient DSB-induced ubiquitylation, providing an alternative means for recruitment of RNF168, one of whose MIUs binds Nedd8 with similar affinity as it binds ubiquitin (Fig 2A). It is, however, somewhat confusing that in the context of UV damage responses, RNF111 appears to act as a conventional ubiquitin ligase (Poulsen *et al*, 2013). Therefore, it remains possible that the stimulatory role of RNF111 in DNA damage-induced ubiquitylation may be a direct consequence of its ubiquitylation activity, instead of being due to its ability to polyneddylate histone H4.

Another recent study describes a DDR role for neddylation that is more in line with its well-characterized function in regulating CRL complexes (Brown *et al*, 2015). Again, Nedd8 modifications were found to stimulate ubiquitylation of chromatin-associated proteins, in this case targeting the essential NHEJ repair factor Ku70/80 (Postow, 2011), probably via neddylation-mediated regulation of an unidentified CRL. Ubiquitylation correlates with removal of the Ku70/80 complex from sites of DNA lesions, and Ku70/80 interactions with VCP/p97 further suggest that this ubiquitin-dependent segregase may be responsible for Ku70/80 extraction from chromatin (Brown *et al*, 2015). These successive events may be important to terminate NHEJ or to allow alternative repair

mechanisms such as HR to take over. Earlier work had already shown Ku80 dispersal from DNA damage sites depending on its polyubiquitylation (Postow *et al*, 2008), which in frog egg extract systems was attributed to the $CRL1^{Skp1/Fbxl12}$ complex (Postow & Funabiki, 2013). Ku80 modification with K48-linked ubiquitin chains and subsequent proteasomal degradation has also been reported for human cells, yet was proposed to depend on RNF8 (Feng & Chen, 2012). It is presently not clear whether the actions of CRL and RNF8 ubiquitin ligases in this case are connected, but since damage-induced neddylation appears to be independent of chromatin-associated ubiquitylation and hence probably independent of RNF8, it is hard to reconcile these findings in a simple model. Nevertheless, it was proposed that a certain ubiquitin modification provided by RNF8 may be needed by $CRL1^{Skp1/Fbxl12}$ for the formation of K48-linked polyubiquitin chains (Postow & Funabiki, 2013).

Deeper insight into the roles of CRL ubiquitin ligase complexes in chromatin-associated ubiquitylation, and hence the link between neddylation and ubiquitylation, in response to DNA damage comes from the mode of action of the $CRL4A^{DBB2}$ complex, which is selectively recruited to UV lesions repaired by NER (Scrima *et al*, 2011). This process shows similarities with protein group SUMOylation, in that $CRL4A^{DBB2}$ ubiquitylates a variety of substrates in direct proximity of damage sites in a rather promiscuous fashion. An explanation for this activity lies in its molecular architecture, which defines a spatially confined zone around the lesion that can be reached by the $CRL4A^{DBB2}$ complex (Scrima *et al*, 2008), combined with its ability to induce chromatin decondensation through recruitment of chromatin remodelers (Luijsterburg *et al*, 2012b; Pines *et al*, 2012). Consistent with a role for Nedd8 in this process, $CRL4A^{DBB2}$ binds to the COP9 signalosome (CSN) complex, which bears deneddylase activity (Groisman *et al*, 2003; Luijsterburg *et al*, 2007). The CSN complex dissociates from $CRL4A^{DBB2}$ upon binding of the ubiquitin ligase complex to DNA lesions, thereby stabilizing the neddylated active form of $CRL4A^{DBB2}$ and unleashing its ubiquitylating activity (Luijsterburg *et al*, 2007). Among the substrates ubiquitylated by $CRL4A^{DBB2}$ are histones H2A (Kapetanaki *et al*, 2006), H3, and H4 (Wang *et al*, 2006), as well as the lesion sensor XPC (Sugasawa *et al*, 2005) and the ligase subunit DDB2 itself (Luijsterburg *et al*, 2007). $CRL4A^{DDB2}$-mediated XPC ubiquitylation enhances its affinity for DNA and may prime XPC for the detection of photolesions in proximity to the docked $CRL4A^{DBB2}$ (Sugasawa *et al*, 2005). However, the fact that $CRL4A^{DDB2}$-mediated ubiquitylation is also required for recruitment of VCP/p97 to damaged chromatin, where it in turn mediates extraction of XPC and DDB2 (Puumalainen *et al*, 2014), complicates the understanding of how protein recruitment and neddylation/ubiquitylation are linked at UV lesions. A clarifying explanation may be the recent observation that the STUbL RNF111 controls XPC extraction by modifying XPC with K63-linked polyubiquitin chains (van Cuijk *et al*, 2015), suggesting that $CRL4A^{DDB2}$ and RNF111 could play successive roles in the process by stimulating recruitment and extraction of XPC, respectively. According to this model, $CRL4A^{DDB2}$ indirectly affects the recruitment of VCP/p97 since it ensures chromatin association of VCP/p97's substrate XPC, whereas RNF111 regulates the actual disposal of XPC by VCP/p97. It is clear, however, that the role of SUMO, Nedd8, and ubiquitin in the regulation of XPC is complex and that additional work is required to decipher the responsible molecular mechanisms.

Poly(ADP-ribose)-guided ubiquitylation

Poly(ADP-ribose) (PAR) is a PTM that consists of at least two but often more ADP-ribose molecules covalently linked by glycosidic ribose–ribose bonds (Leung, 2014). The human genome encodes a family of enzymes that can modify proteins with mono(ADP-ribose) or PAR, with the abundant nuclear protein poly(ADP-ribose) polymerase 1 (PARP1) being responsible for the bulk of PARylation at DNA lesions (Langelier & Pascal, 2013). Through their self-organizing nucleation property, PAR chains facilitate the recruitment of specific proteins to a variety of DNA lesions such as photolesions, SSBs, and DSBs (Hakme *et al*, 2008). PARP1 is recruited to DSBs by means of two zinc fingers and a WGR domain of unknown function (Langelier *et al*, 2011; Ali *et al*, 2012). This results in rapid PARP1 activation and PARylation of various proteins at sites of DNA damage, among them prominently PARP1 itself. Importantly, damage-induced PARylation at DSBs is much more transient in nature than phosphorylation and ubiquitylation, a phenomenon that may at least in part be due to the relatively short half-life of PAR protein modifications (Hakme *et al*, 2008).

The ubiquitin ligase CHFR combines an N-terminal FHA domain with a RING domain, a molecular architecture that it shares with RNF8. CHFR is also recruited to DNA damage but, unlike RNF8, its recruitment depends neither on its FHA domain nor on ATM-mediated histone H2AX phosphorylation. Instead, CHFR recruitment occurs through a direct interaction between PAR chains and a conserved zinc finger motif in its C-terminal region (Oberoi *et al*, 2010) (Fig 2B). As such, CHFR can be considered a PAR-targeted ubiquitin ligase that directly links the ubiquitylation response to the instant and transient wave of DNA damage-induced PARylation. In line with this idea, CHFR has been found to be involved in the early ubiquitylation response at sites of DSBs, where it modifies auto-PARylated PARP1 with K48- and K63-linked ubiquitin chains (Liu *et al*, 2013). This results in chromatin extraction and degradation of PARP1, providing another means to temporally confine the PARylation response to the early stage of the DDR. This mode of action of CHFR is not limited to DNA damage sites, since mitotic stress conditions also induce autoPARylation-dependent ubiquitylation and degradation of PARP1, thereby initiating mitotic arrest in prophase (Kashima *et al*, 2012). Studies with knockout mice showed that RNF8 and CHFR play synergistic roles in the DDR by regulating ubiquitylation of core histones H2A and H2B (Wu *et al*, 2011). This in turn leads to reduced histone acetylation, which may be the primary cause for the inability of cells lacking RNF8 and CHFR to mount a robust ATM response. Genetic ablation of RNF8 combined with CHFR sensitizes mice to ionizing radiation and results in the development of T-cell lymphoma, underscoring the importance of the combined action of these phospho- and PAR-targeted ubiquitin ligases in the DDR (Wu *et al*, 2011).

RNF146 (also known as Iduna) is another RING domain ubiquitin ligase that specifically targets proteins that are marked by PARylation. In this case, a WWE domain present in RNF146 mediates its interaction with PARylated substrates (Sarangi & Zhao, 2015). Since WWE domains are present in various other ubiquitin ligases, the phenomenon of PAR-dependent protein–protein interactions could be widespread among ubiquitin ligases. Remarkably, the WWE domain is not only required for RNF146 recruitment, since its interaction with PAR chains also has a stimulatory effect on the ubiquitin ligase activity of RNF146 (Wang *et al*, 2012).

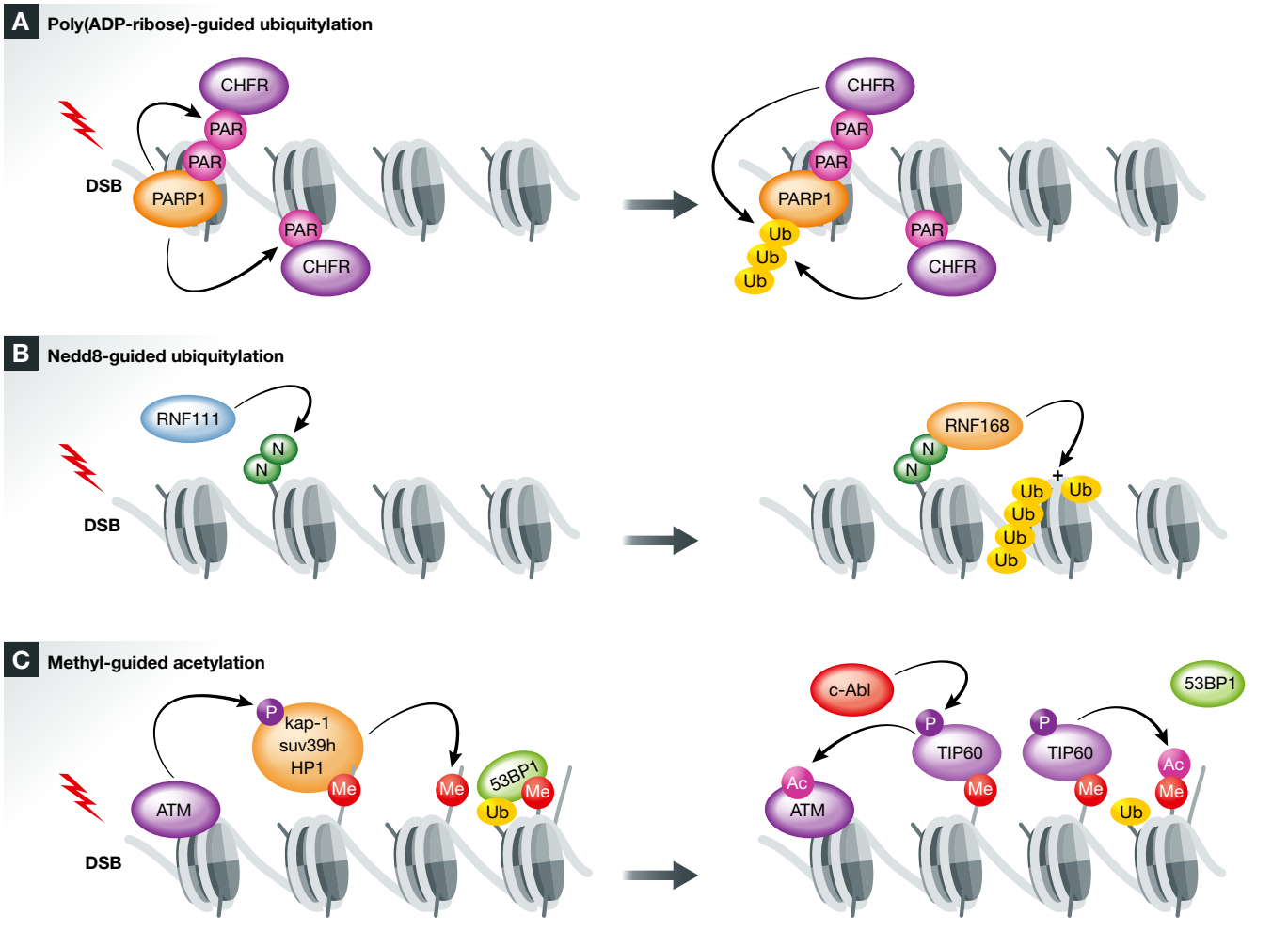


Figure 2. PTM-guided PTMs in the DDR (part 2).
Schematic representation of (A) Poly(ADP-ribose)-guided ubiquitylation, (B) Nedd8-guided ubiquitylation, and (C) methyl-guided acetylation. See sections "Poly(ADP-ribose)-guided ubiquitylation", "Nedd8-guided ubiquitylation" and "Methyl-guided acetylation" for more details. Ac, acetyl group; HMTs, histone methyltransferases; Me, methyl group; N, Nedd8 moiety; PAR, poly(ADP-ribose) group; P, phosphate group; Ub, ubiquitin moiety.

Structural data show that in the absence of PAR chains, the WWE domain blocks the interaction between RNF146 and its cognate ubiquitin-conjugating enzyme (DaRosa *et al*, 2015). The interaction between PAR chains and RNF146 is required for DNA damage-induced ubiquitylation and subsequent proteasome-dependent degradation of a range of proteins involved in signaling and repair of DNA damage, including histones, Ku70, as well as PARP1 and PARP2 (Kang *et al*, 2011).

The number of domains mediating PAR recognition is further expanded with the finding that FHA and BRCA1 C-terminal (BRCT) domains, best known for their specific binding to phospho-peptides, can also interact with PAR chains (Li *et al*, 2013). To our knowledge, it is unclear whether this is because of similarities in structure, or negative charge shared between PAR moieties and phospho-groups. The interaction between BRCT domains in BARD1 and PAR chains regulates the early recruitment of the BRCA1/BARD1 complex to sites of DNA damage (Li & Yu, 2013). Since the ubiquitin ligase activity of BRCA1 was reported to matter for cells to resist ionizing radiation (Ruffner *et al*, 2001), BRCA1 may thus also be considered a PAR-targeted ubiquitin ligase. However, other work could not confirm such a role for BRCA1's ligase activity in DNA repair and the protection against genotoxic agents, indicating that more research is still needed to solidify a role for the ubiquitin ligase activity of BRCA1 in the DDR (Reid *et al*, 2008).

Methyl-guided acetylation

While the list of PTMs induced by ubiquitylation, neddylation, SUMOylation, or PARylation is already extensive, this repertoire is further expanded by reports on the importance of crosstalk between histone marks for fostering a proper DSB response, in this case lysine 9 trimethylation of histone H3 (H3K9me3) and lysine 16 acetylation of histone H4 (H4K16ac) (Fig 2C). Collectively, this work suggests that a complex consisting of KAP1, HP1, and the H3K9 methyltransferase Suv39h1 is rapidly loaded at DSBs. Suv39h1 trimethylates histone H3 at lysine 9, allowing binding and spreading of additional KAP1/HP1/Suv39h1 complexes to nascent

H3K9me3 marks via KAP1's chromodomain throughout the DSB-flanking chromatin (Ayrapetov *et al*, 2014). Importantly, recruitment of activated ATM leads to phosphorylation of KAP1 and the subsequent release of this complex from H3K9me3, providing a negative feedback loop that frees H3K9me3 modifications for binding by other proteins. Indeed, the acetyltransferase TIP60/KAT5 then binds this mark through its chromodomain (Sun *et al*, 2009; Ayrapetov *et al*, 2014), and this interaction is further enhanced by damage-induced TIP60 phosphorylation through the proto-oncogenic kinase c-Abl (Kaidi & Jackson, 2013). Once bound to H3K9me3, TIP60 acetylates both ATM and histone H4 at lysine 16. Acetylation of ATM has been shown to enhance further ATM activation, ATM-mediated checkpoint signaling and repair, as well as cell survival in response to DNA damage (Sun *et al*, 2009; Kaidi & Jackson, 2013; Ayrapetov *et al*, 2014). On the other hand, H4K16ac has been shown to preclude Tudor domain-mediated binding of 53BP1 to the adjacent dimethyl mark at H4K20 (Hsiao & Mizzen, 2013; Tang *et al*, 2013). Together, these findings indicate an important role for distinct methyl-guided acetylation events that regulate the DSB response at the level of ATM and 53BP1 function.

Functional significance of serial, parallel, and combinatorial PTMs

While each of the chromatin-associated PTMs that are induced by DNA damage is of great importance, it is the variety of PTMs that provides another critical layer of complexity to the DDR. Not only do they determine when and where specific proteins are being recruited, but they also ensure that proteins are removed from chromatin in a timely fashion upon completion of repair. Impeding either proper recruitment or extraction of proteins is known to hamper the cell's ability to cope with genomic stress conditions, suggesting that both these processes are central in the DDR. In the next few sections, we will discuss some paradigms illustrating how DNA damage-induced PTMs form a functional platform that increases specificity, reduces background activity, and provides the level of temporal and spatial resolution needed for proper activation and termination of the DDR.

Serial PTMs

As shown by live-cell imaging, the order in which proteins are recruited to DSBs reflects the sequential appearance of PTMs responsible for their recruitment. For instance, MDC1 and RNF8, recruited via selective binding to phospho-modifications generated by ATM, are first detected at DSBs (Mailand *et al*, 2007). They are immediately followed by the ubiquitin ligase RNF168 that binds ubiquitin conjugates generated by RNF8 (Doil *et al*, 2009). This in turn is followed by the localization of proteins interacting with ubiquitin conjugates generated by RNF168, such as 53BP1 and BRCA1 (Fig 3A). BRCA1 itself forms a ubiquitin ligase complex together with its binding partner BARD1 (Polanowska *et al*, 2006), which has been found to modify histone H2A at lysine residues (K127 and K129) differing from those targeted by RNF168 (Kalb *et al*, 2014). The chain of events may therefore continue even further to promote a sequential recruitment of proteins.

Spatiotemporally different PTM patterns at sites of DNA damage are particularly evident when comparing damage-induced PARylation, phosphorylation, and ubiquitylation. The mechanistic peculiarities of PARylation result in a spatial and temporal PAR distribution that is quite distinct from phosphorylation of H2AX, despite the fact that both are triggered almost simultaneously in response to DSBs. DNA damage initiates massive but also very transient PARylation in the direct vicinity of the lesion (Beck *et al*, 2014), whereas γH2AX spreads away from the damage site and decorates chromatin in a DSB-flanking region encompassing up to 1.7 Mb of DNA, persisting up to several hours (Iacovoni *et al*, 2010). PARylation involves various self-confining mechanisms, such as the charge-dependent DNA-repelling nature of PAR chains, extraction of PARP1 by PAR-targeted ubiquitin ligase, and hydrolysis of PAR chains by the glycohydrolase PARG (Pines *et al*, 2013). The first two inhibitory mechanisms are directly linked to DNA damage-induced PARylation, explaining the inherent transient nature of the PARylation response. Despite the fact that the dynamics of, for example, phosphorylation, ubiquitylation, and PARylation are very different, they are not mutually exclusive, and it is conceivable that at the time and place where these signals co-occur they may, as discussed below, provide a higher-order "barcode" that facilitates recruitment of a unique set of proteins.

The different spatial and temporal distributions of these PTMs are likely relevant for setting up a proper chromatin environment for early and late events during the signaling and repair of DNA damage. PARylation has been shown to be required for DSB recruitment of several chromatin remodeling enzymes, including ALC1 (Ahel *et al*, 2009; Gottschalk *et al*, 2009), CHD4 (Chou *et al*, 2010; Polo *et al*, 2010; Luijsterburg *et al*, 2012a), and SMARCA5/SNF2H (Smeenk *et al*, 2013). Moreover, PARylation can trigger chromatin decondensation at sites of UV damage by enforcing recruitment of ALC1 through stabilization of DDB2, the CRL4 ubiquitin ligase adaptor involved in UV damage recognition (Luijsterburg *et al*, 2012b; Pines *et al*, 2012; Robu *et al*, 2013). Recent work showed that PARylation can also induce local phase separation at DSBs, allowing DDR proteins with intrinsically disordered domains to assemble at lesion while at the same time temporarily excluding other DDR proteins (Altmeyer *et al*, 2015). This may, in addition to the recruitment of chromatin remodelers, be yet another means by which PAR chains control the local environment at damage sites to ensure a proper DDR. Given their amplification and spreading via positive feedback mechanisms, DNA damage-induced phosphorylation and ubiquitylation, on the other hand, are able to provide a powerful and more persistent signal for mobilizing proteins that facilitate DNA repair and signaling. At the same time, their sheer magnitude can also generate a pool of binding sites functioning as a "sink" for sequestration of repair proteins distal to DSBs. This can paradoxically lead to functional inhibition of processes such as HR, as has been proposed for sequestration of the BRCA1/RAP80 complex by binding to K63-linked ubiquitin chains at the surrounding chromatin (Hu *et al*, 2011).

Proteins that are recruited to sites of DNA lesions have to be removed in a timely manner so as to allow subsequent steps in the response to take place or, alternatively, to terminate the response. It has become increasingly clear that, rather than being a passive consequence of the reversal of a retention signal, removal of proteins is often an active and highly coordinated process also involving regulation by PTMs (Dantuma *et al*, 2014). It is evident

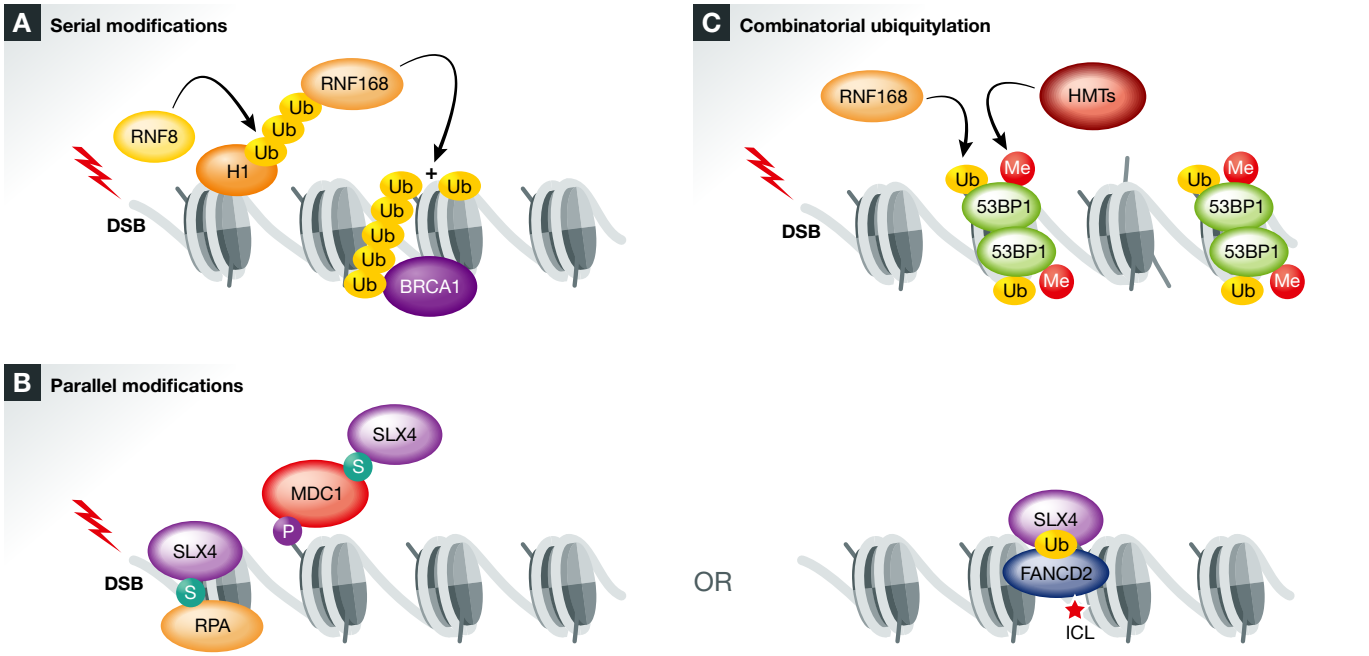


Figure 3. Serial, parallel, and combinatorial PTMs in the DDR.
Schematic representation of examples involving (A) different ubiquitin modifications as serial PTMs, (B) SUMOylation and ubiquitylation as parallel PTMs, and (C) methylation and ubiquitylation as combinatorial PTMs. See sections "Serial PTMs", "Parallel PTMs", and "Combinatorial PTMs" for more details. Me, methyl group; P, phosphate group; S, SUMO moiety; Ub, ubiquitin moiety.

that the order in which given PTMs appear, as well as the lag time between the establishment and the final reversal of the modification, is critical in such processes. The multistep pathway leading to ubiquitin-dependent extraction of RNF4 substrates provides a good example of the regulatory power of serially organized PTMs. Initial recruitment of the RNF4 substrate MDC1 is facilitated by histone H2AX phosphorylation (Stewart *et al*, 2003), a process that at the same time sets off DNA damage-induced ubiquitylation by the RNF8/RNF168 pathway (Huen *et al*, 2007; Kolas *et al*, 2007; Mailand *et al*, 2007). As discussed above, this ubiquitylation in turn stimulates local SUMOylation of a number of targets including MDC1, resulting in recruitment of the STUbL RNF4 by binding to polySUMO chains on MDC1, and mediating MDC1 polyubiquitylation to trigger its removal from the chromatin (Galanty *et al*, 2012; Luo *et al*, 2012; Yin *et al*, 2012). Failure of timely MDC1 removal inhibits efficient DSB repair, suggesting that MDC1 dispersal may be required for the recruitment of downstream factors (Galanty *et al*, 2012; Luo *et al*, 2012; Yin *et al*, 2012). It is interesting that the phosphorylation signal that plays a central role in the recruitment of MDC1 at the same time triggers the sequence of events that results in its disposal, suggesting that this PTM determines the delay between recruitment and extraction of MDC1 at DSBs. Targeting of PTMs that occur in-between the recruitment and removal may thus be a possible means of modulating the residence time of MDC1 in response to external cues. Similar mechanisms may be in play in the regulation of other proteins that are actively being removed from the chromatin in a DNA damage-induced fashion (Acs *et al*, 2011; Galanty *et al*, 2012; Mallette *et al*, 2012; Yin *et al*, 2012; Brown *et al*, 2015).

Parallel PTMs

Proteins that are recruited to DNA damage sites often contain binding domains for multiple PTMs. An example of a protein relocating to DNA lesions via at least two non-redundant recruitment mechanisms is the above-mentioned SNF2-like ATPase CHD4, the catalytic subunit of the repressive chromatin remodeling complex NuRD (Xue *et al*, 1998). One recruitment mechanism depends on DNA damage-induced PARylation (Chou *et al*, 2010; Polo *et al*, 2010; Smeenk *et al*, 2010). This process has been shown to be important for removing RNA polymerase II from sites of DNA damage. It was therefore proposed that the PAR-recruited NuRD complex facilitates the latter process by imposing a transient repressive chromatin state at DNA lesions, although a causal CHD4 contribution to the removal of RNA polymerase II still needs to be established (Chou *et al*, 2010; Polo *et al*, 2010). In a second, apparently unrelated process, CHD4 stimulates DNA damage-induced ubiquitylation at the level of RNF8. Accordingly, depletion of CHD4 impairs ubiquitin-dependent recruitment of BRCA1 to DSBs (Larsen *et al*, 2010; Polo *et al*, 2010; Smeenk *et al*, 2010; Luijsterburg *et al*, 2012a). The stimulatory function of CHD4 in DNA damage-induced ubiquitylation is, however, independent of its PAR-dependent recruitment, but instead relies on direct CHD4 interaction with RNF8 whereby it stimulates RNF8-mediated ubiquitylation of chromatin-associated proteins (Larsen *et al*, 2010; Smeenk *et al*, 2010; Luijsterburg *et al*, 2012a). This pool of CHD4, which is functionally different from the PAR-recruited CHD4, induces chromatin decondensation, making the local chromatin environment amenable for RNF8-induced ubiquitin modifications (Luijsterburg *et al*, 2012a). Importantly, the functional difference between PAR-dependent and RNF8-dependent

recruitment of CHD4 underscores that distinct molecular mechanism responsible for recruitment may be able to dictate a protein's action at sites of DNA damage.

The divergent roles of ubiquitin and SUMO2/3 in the translocation of Slx4, a scaffold protein that interacts with DNA structure-specific endonucleases (Andersen *et al*, 2009; Fekairi *et al*, 2009; Munoz *et al*, 2009; Svendsen *et al*, 2009), are another example of how different PTMs can unleash different functions in a given protein, and activate distinct DNA repair pathways (Fig 3B). In addition to two ubiquitin-binding zinc finger (UBZ) domains that are important for its function in DNA interstrand cross-link (ICL) repair (Yamamoto *et al*, 2011; Lachaud *et al*, 2014), recent studies revealed that Slx4 also contains a cluster of three SIMs used to interact with SUMO2/3 conjugates at DNA lesions (Guervilly *et al*, 2015; Ouyang *et al*, 2015). The UBZ domains, while being critical for Slx4 interaction with monoubiquitylated FANCD2 during ICL repair, are dispensable for Slx4 localization to DSBs inflicted by laser damage. On the contrary, the SIM cluster does not seem to contribute to ICL repair, but is of key importance for the recruitment of Slx4 to DSBs, where it interacts with MDC1 and, in an RPA-dependent fashion, with resected DNA ends. However, when DNA damage other than ICLs is inflicted during DNA replication, both the UBZ and SIMs are required for Slx4 recruitment. Finally, PARylation also contributes to Slx4 recruitment to laser-inflicted DNA damage, although it is presently unclear what type(s) of lesion this recruitment can be attributed to and whether there is overlap with ubiquitin and SUMO binding at these sites (Gonzalez-Prieto *et al*, 2015). In conclusion, these studies demonstrate that deconvolution of the roles of multifunctional DNA repair proteins can be a key function of PTMs involved in independent recruitment mechanisms.

As interactors of ATM/ATR kinase-generated phospho-threonine and phospho-serine modifications, FHA and BRCT domains are intimately linked to the DDR (Mahajan *et al*, 2008). More recently, however, some FHA and BRCT domains were also found to bind PAR chains generated at DNA lesion (Li *et al*, 2013). Of particular interest is the BRCA1/BARD1 ubiquitin ligase complex, whose subunits both contain BRCT domains. While BRCA1/BARD1 is recruited to RNF8/RNF168-induced ubiquitin moieties at DSBs through its interaction with the ubiquitin-binding protein RAP80 (Huen *et al*, 2007; Kolas *et al*, 2007; Mailand *et al*, 2007), one of the BRCT domains in BARD1 can mediate fast sequestration at these lesions by binding to locally synthesized PAR chains (Li & Yu, 2013). Whether these two distinct recruitment BRCA1/BARD1 mechanisms are complementary and simply ensure that the complex is similarly present at different stages of the response, or whether the BRCA1/BARD1 complex has indeed differential roles depending on the mode/timing of recruitment remains to be determined.

Finally, additional factors may also rely on alternative recruitment mechanisms depending on the type of damage. As discussed earlier, whereas the γH2AX-dependent recruitment of RNF8 in response to DSBs is well documented (Huen *et al*, 2007; Kolas *et al*, 2007; Mailand *et al*, 2007), RNF8 also translocates to UV-induced damage to initiate a similar ubiquitylation response that culminates in the recruitment of BRCA1 and 53BP1 (Marteijn *et al*, 2009). The fact that their recruitment to UV lesion occurs independently of ATM/ATR-dependent γH2AX formation (Bergink *et al*, 2006), but still requires ATR kinase and the γH2AX interactor MDC1, suggests that MDC1 and consequently RNF8 are recruited to UV lesion by a yet unknown modification distinct from γH2AX (Marteijn *et al*, 2009).

Combinatorial PTMs

Multivalent interactions with nucleosomes provide a powerful means to increase the avidity of proteins for specific chromatin landscapes. This concept also applies to the DDR, where a bivalent recruitment mechanism involving both histone methylation and ubiquitylation has been described for the tumor suppressor 53BP1, a protein that regulates DSB repair pathway choice (Panier & Boulton, 2014) (Fig 3C). The tandem Tudor domain of 53BP1 specifically "reads" a dimethyl mark at lysine 20 of histone H4 (H4K20me2) (Botuyan *et al*, 2006). At the same time, the 53BP1 carboxy-terminal region acts as an atypical ubiquitin-binding domain, which instead of exhibiting restricted interaction with conjugated ubiquitin specifically recognizes lysine 15-ubiquitylated histone H2A (H2AK15ub), the unique mark generated by RNF168 (Mattiroli *et al*, 2012). This ubiquitin-dependent recruitment motif (UDR) in 53BP1 appears to recognize an epitope formed by H2AK15ub together with the lysine 15-flanking residues in the histone N-terminal tail (Fradet-Turcotte *et al*, 2013). The multivalent nature of the interaction provides additional layers of control to either support or repress stable tethering of 53BP1. The RNF8/RNF168 pathway is not only required to generate the particular H2AK15ub modification, but also—albeit less directly—helps in establishing the 53BP1–H4K20me2 interaction. VCP/p97, which is recruited downstream of RNF8 action, triggers selective chromatin extraction of L3MBTL1, a polycomb protein that itself binds (and thus masks) the H4K20me2 mark required for 53BP1 recruitment (Acs *et al*, 2011). Furthermore, another H4K20me2 interactor, the histone demethylases JMJD2A, is removed from chromatin upon DNA damage in an RNF8-dependent fashion (Mallette *et al*, 2012). Regulating accessibility of the H4K20me2 mark during the DSB response appears to be a broader concept that is not limited to ubiquitin-dependent events, given the emerging role of H4K16 acetylation in sterically blocking 53BP1 binding to adjacent H4K20me2 marks (discussed earlier in section "Methyl-guided acetylation") (Hsiao & Mizzen, 2013; Tang *et al*, 2013). Finally, 53BP1 itself has been found to be directly ubiquitylated by RNF168, resulting in 53BP1 oligomerization, a process that is critical for its relocalization to DSBs (Lottersberger *et al*, 2013).

An interesting twist involving yet another PTM can be found in the mechanism that represses 53BP1 recruitment to DSBs during mitosis. While the initial DSB response steps, such as ATM activation, H2AX phosphorylation, and MDC1 recruitment, also operate in dividing cells, recruitment of RNF8 and downstream factors is strongly suppressed (Giunta *et al*, 2010) as a consequence of RNF8 and 53BP1 phosphorylation by mitotic kinases (Orthwein *et al*, 2014). In the case of 53BP1, this has been attributed to two phosphorylation sites within its UDR that, when phosphorylated, suppress recognition of the H2AK15ub modification (Lee *et al*, 2014; Orthwein *et al*, 2014). Consequently, efficient 53BP1 recruitment to allow functional NHEJ repair during the subsequent G1 phase of the cell cycle requires specific dephosphorylation of these two residues by the PP4C/R3β phosphatase (Lee *et al*, 2014). Given 53BP1's key role in determining whether cells will activate NHEJ or HR repair, its multilayered recruitment process being dependent on methylation,

ubiquitylation, phosphorylation, and acetylation provides an important regulatory mechanism for DSB repair.

It is tempting to speculate that combinatorial PTMs play a more general role in guiding the specificity of the cellular response to DNA damage. Interestingly, some PTMs induced by DNA damage appear to more promiscuously decorate various chromatin-associated targets primarily due to their proximity to the lesion. This generates robust signals that are not exclusive to the DDR but also occur in the context of other chromatin-associated events. Their merely quantitative nature raises the question how they are translated into the qualitative information that indicates the presence of specific DNA lesions. For example, this is relevant for the earlier discussed proposal of SUMO group modification, as opposed to site-specific SUMOylation of certain well-defined targets, acting as a topological trigger activating an appropriate DDR (Jentsch & Psakhye, 2013). Proteomic analyses identified a broad variety of DNA damage-induced SUMO2 substrates, consistent with such a model of unleashing a general SUMOylation activity that targets proteins based on proximity rather than sequence specificity (Hendriks *et al*, 2015). Similarly, PARylation also affects a large number of proteins found in the vicinity of DNA lesions (Jungmichel *et al*, 2013). Other examples include the RNF8/RNF168 pathway, which generates a profound accumulation of ubiquitin conjugates at DNA lesions (Bekker-Jensen & Mailand, 2011), and the $CRL4^{DDB2}$ ubiquitin ligase, which may define a ubiquitylation zone in direct proximity to UV damage sites (Scrima *et al*, 2011). The rather promiscuous nature of these modifications affecting multiple locally confined regions begs the question of how cells can still derive useful information from such signals of seemingly limited specificity. In addition, chromatin-associated SUMOylation, PARylation, and ubiquitylation are by no means exclusive to the DDR but also prominently found in other contexts such as transcription or DNA replication (Muratani & Tansey, 2003; Hay, 2005; Kraus, 2008). It is therefore conceivable that information resides less in the signals themselves, but rather in the complex combination of such PTMs. Proteins relying (either directly or indirectly) on combinatorial PTMs for their recruitment may thus function as readers that disambiguate and limit activation of certain pathways or branches to situations where appropriate signal combinations are presented.

Another type of a bivalent interaction module relies on simultaneous binding to ubiquitin chains and adjacent chromatin-associated motifs. For example, RAP80 can preferentially recognize K63-linked polyubiquitin chains through the structural spacing of its tandem ubiquitin-binding motifs in RAP80 (Sato *et al*, 2009; Sims & Cohen, 2009), but given that K63-linked ubiquitin also occurs in other cellular processes, this cannot singly explain specific RAP80 accumulation at DSBs (Komander & Rape, 2012). It turns out that the ubiquitin-binding domains in RAP80 are not sufficient for its translocation, but additionally require a so-called LR motif located adjacent to the ubiquitin-binding domain, which stabilizes RAP80 chromatin association (Panier *et al*, 2012). Interestingly, artificially optimizing the RAP80 ubiquitin-binding module, by adjusting the spacer length between the ubiquitin-binding domains (Sims & Cohen, 2009), abrogates the need for the adjacent LR motif (Panier *et al*, 2012).

Several other DDR proteins, including RNF168 and RNF169, also employ LR motifs for context-specific ubiquitin binding (Panier *et al*, 2012). RNF168 employs its N-terminal ubiquitin-binding module for binding the ubiquitin conjugates generated by RNF8, while the ubiquitin-binding site present in its C-terminal region is important for accumulation at RNF168-generated ubiquitin conjugates, establishing a positive feedback loop for signal amplification and a second wave of RNF168-mediated ubiquitylation (Panier *et al*, 2012). A recent study indicates that an N-terminal module of RNF168 functions as a specific reader of K63-ubiquitylated histone H1 generated by RNF8 and Ubc13 (Thorslund *et al*, 2015). The different specificities of RNF168's ubiquitin-binding modules for RNF8- vs. RNF168-generated ubiquitin chains result from adjacent LR motifs interacting with chromatin-associated ligands (Panier *et al*, 2012). In contrast, RNF169 cannot bind RNF8-induced ubiquitin conjugates, but contains an LR motif that facilitates recognition of ubiquitin conjugates assembled by RNF168 (Panier *et al*, 2012). As such, RNF169 does not interfere with the initial activation of the DDR, but selectively competes with recruitment of other DDR proteins to attenuate the second wave of chromatin-associated ubiquitylation and accumulation of RAP80 and 53BP1 (Chen *et al*, 2012; Poulsen *et al*, 2012). The divergent specificities of the bipartite ubiquitin/chromatin binding modules in RNF168 and RNF169 demonstrate the importance of context-dependent PTM recognition and suggest that such modules can be used for specific spatial and temporal coding of protein localization.

Interactions between DNA damage-associated ubiquitin ligases and nucleosomes offer another well-established concept of multivalent binding determinants involving additional accessory protein interaction motifs. A triad of acidic residues in the sequences of histones H2A and H2AX is critical for DNA damage-induced histone ubiquitylation (Chen *et al*, 2013). This acidic triad, in combination with another region on histone H2B, forms a negatively charged patch that interacts with the RING domain of RNF168, explaining why the H2A-H2B dimer is the minimal unit needed for selective ubiquitylation by RNF168 (Mattiroli *et al*, 2014). Importantly, efficient DNA damage-induced ubiquitylation requires bivalent RNF168–nucleosome interactions mediated by the MIU domains through binding ubiquitin conjugates and the RING domain through binding to the H2A-H2B acidic patch, respectively (Leung *et al*, 2014). The same acidic patch is also utilized for recruitment of polycomb recessive complex (PRC) 1 (Leung *et al*, 2014), which mediates lysine 119 monoubiquitylation of H2A in the vicinity of DNA lesions (Bergink *et al*, 2006; Ginjala *et al*, 2011; Ismail *et al*, 2012). Here, structural analyses revealed the contribution of an arginine residue in the Ring1b subunit in anchoring PRC1 to the acidic patch and hence to chromatin (McGinty *et al*, 2014). At the same time, several PTMs have been linked to PRC1 chromatin recruitment, such as PRC2-dependent lysine 27 trimethylation of histone H3 (H3K27me3) (Fischle *et al*, 2003; Min *et al*, 2003), as well as SUMOylation (Ismail *et al*, 2012) and PARylation (Chou *et al*, 2010), suggesting that the interaction between PRC1 and nucleosomes may also be of combinatorial nature. In addition, PRC1 localization to DNA lesions involves the ATM-phosphorylated transcription elongation factor ENL (Ui *et al*, 2015) and is stimulated by the chromatin remodeler PBAF (Kakarougkas *et al*, 2014). To ensure repair of actively transcribed regions of the genome, PRC1 gives rise to local repression of transcription at sites of DNA damage (Shanbhag *et al*, 2010), a process that obviously needs to be tightly regulated and confined to only the damaged region of

transcribed DNA. Likewise, the STUbL RNF4 utilizes a multivalent recruitment mechanism, binding not only to chromatin-associated SUMO2/3 conjugates but also establishing direct interactions with nucleosomes via a basic patch within its RING domain (Groocock *et al*, 2014). Thus, the minimal targeting system of RNF4 also relies on the combination of a PTM and the presence of nucleosomes, although it is presently not clear whether this involves the same acidic patch on H2A/H2B that is recognized by RNF168 and PRC1. The recurrence of bivalent interactions in the recognition of PTMs on nucleosomes implies that this may represent a general mechanism for histone modifiers to target specific PTMs only when they are found in the correct chromatin context.

Concluding remarks

Intracellular signaling cascades that rely on posttranslational modification of target proteins are of critical importance to mount an appropriate cellular response to both extracellular and intracellular cues. As such, also the cellular response to genotoxic insults is tightly regulated by a multitude of PTMs that target chromatin or chromatin-associated proteins in proximity to sites of DNA damage. During recent years, it has become clear that DNA lesions are decorated with a variety of PTMs that are generated and reverted in a highly ordered manner. It is also evident that a considerable amount of crosstalk exists between these modifications, giving rise to a DDR driven by serial, parallel, and combinatorial PTMs. Despite the large number of different modifications that are involved, none of them is unique to the DDR and it remains to be elucidated which critical information allows cells to distinguish PTMs generated at damaged chromatin from the exact same PTMs present in other, intact chromosomal regions. Moreover, cells need to ensure stepwise execution of DDR processes in time, and while it is clear that the same assortment of PTMs provides essential information in this respect, it remains puzzling how PTMs integrate spatial and temporal information. Thus, DNA damage-induced PTMs have to provide high-resolution spatiotemporal information at sites of DNA damage to ensure that recruitment of proteins occurs in the correct order and at the right position. Possible explanations may lie not only in the number of different modifications involved in the DDR, but also in their specific sequence of appearance, which will change the PTM landscape on chromatin before, during, and after DNA damage repair. Furthermore, the combination of signals at DNA lesions at any given time may offer important information since "barcode reading" of such combinatorial signals may allow distinguishing DNA damage marks from similar PTMs at other chromatin regions. Overall, the plethora and their increasing complexity of PTMs in the DDR has made clear that cells leave little of their molecular signaling repertoire unused when it comes to ensuring a solid and reliable activation of damage response and repair pathways, in order to maximize chances to survive under conditions of genotoxic stress. Nevertheless, much remains still to be learned about the fascinating and sophisticated paradigm of the PTM-driven cellular response to DNA damage.

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Conflict of interest

The authors declare that they have no conflict of interest.

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